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Characterisation of Multiple Glutathione Transferases Containing the GST I Subunit with Activities toward Herbicide Substrates in Maize (*Zea mays*)*

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Abstract: The glutathione transferases (GSTs) of maize with activities toward chloroacetanilide herbicides are relatively well characterised, but their range of substrate specificities has not been determined in detail. GST activities toward an extensive range of chemically diverse xenobiotic substrates, including the herbicides atrazine, alachlor, metolachlor and fluorodifen, have been determined in crude and purified preparations from the roots and shoots of dark-grown maize seedlings treated with and without the herbicide-safener dichlormid. With the exception of the activity toward atrazine, specific activities were higher in the roots than in the shoots in all cases. In untreated shoots activities were in the order atrazine = alachlor = metolachlor > fluorodifen with safener–treatment selectively increasing the activity toward the chloroacetanilides and fluorodifen. In the roots the highest GST activities toward herbicides were toward the chloroacetanilides. Dichlormid treatment resulted in an increase in activities toward all four herbicides in the roots of one maize cultivar (Pioneer 3394) but only enhanced the activities toward the chloroacetanilides and fluorodifen in cultivar Artus. Using the non-herbicide 1-chloro-2,4-dinitrobenzene (CDNB) as substrate, anion-exchange chromatography showed that the roots and shoots contained a similar range of GST isoenzymes. All of these isoenzymes were enhanced in response to safeners, though the extent of this induction was organ-dependent. GST isoenzymes containing the GST I subunit were purified from safener-treated roots by a combination of hydrophobic interaction chromatography and affinity chromatography using Orange A agarose. Three isoenzymes could then be purified following resolution by anion-exchange chromatography. The three GSTs were termed GST I/I, GST I/II and GST I/III with GST I, II and III referring to the presence of 29 kDa, 27 kDa and 26 kDa subunits respectively. This revised nomenclature for the maize GSTs was considered necessary in view of the continued discovery of new isoenzymes, such as GST I/III, composed of subunits which have been previously described. GST I/I had measurable activity toward atrazine, low activities toward the other herbicides and appreciable activities toward a range of other xenobiotic substrates. GST I/II and the novel GST I/III isoenzymes both showed high activities toward the chloroacetanilides and fluorodifen but lower activities toward the other substrates and negligible activities toward atrazine. The GST II subunit of GST I/II also had activity as a glutathione peroxidase. Our results show that the GST I subunit can form dimers with the GST III subunit in addition to the GST I and GST II subunits and that the degree of specificity toward herbicide substrates of the respective isoenzymes is greater than previously reported. Our results also suggest that the safener-inducible GST II subunit has additional activities as a glutathione peroxidase.

Key words: glutathione transferase, herbicides, isoenzymes, substrate specificity, *Zea mays*

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1 INTRODUCTION

Glutathione transferases (EC 2.5.1.18), also termed glutathione *S*-transferases (GSTs), facilitate the detoxification of electrophilic substrates by catalysing nucleophilic substitution or addition reactions with the tripeptide glutathione (GSH). GSTs normally consist of two subunits each with molecular masses in the range 23–30 kDa and each with an active site for both GSH and the electrophilic substrate.[1] Several important classes of herbicides contain electrophilic groups and their metabolism by GSH conjugation in plants following the action of GSTs is well established.[2] Crops such as maize possess a complex content of highly active GSTs which can detoxify a range of herbicides including the chloro-*s*-triazines, the chloroacetanilides and the sulfoxide derivatives of thiocarbamates.[1,2]

Due to their importance in conferring tolerance to these herbicides, the GSTs of maize have been the subject of several studies.[3] Early investigations concentrated on the GSTs in maize responsible for detoxifying atrazine and other chloro-*s*-triazine herbicides. A GST with such an activity was subsequently purified, although its relationship to the other GST isoenzymes in maize with activities toward other herbicide substrates has not been defined.[4] Instead, the majority of effort has been directed toward characterising maize GSTs which detoxify the chloroacetanilide herbicides alachlor and metolachlor. Initial studies with alachlor by Mozer *et al.*[5] identified a major constitutive form of GST composed of two 29 kDa subunits, termed GST I, and a GST composed of a 29 kDa and a 27 kDa subunit, which accumulated in response to treatment with a range of herbicide safeners, which was termed GST II. Herbicide safeners increase the tolerance of maize to a range of herbicides and this is typically associated with increased rates of detoxification including conjugation with GSH.[1–3] An additional constitutive form of GST, composed of two 26 kDa subunits, with high activities toward alachlor was later determined in maize by O'Connell *et al.*[6] This isoenzyme has not been well characterised, but seems to be composed of subunits encoded by a maize cDNA termed GST III.[7,8] These studies have recently been extended with the discovery of a fourth GST isoenzyme with high activities toward chloroacetanilides, termed GST IV, which, in the tissues studied, was only expressed in response to safener treatment and was composed of two 27 kDa subunits.[9] The 27 kDa subunit of GST IV is identical to the 27 kDa subunit in GST II.[10,11] The DNA sequences encoding GST I, GST II and GST III have all been identified,[7,8,10,11] though recent studies have suggested that maize contains at least five distinct genes encoding GSTs[12] and isoenzyme analysis with multiple substrates has suggested that safener-treated maize shoots contains up to seven distinct GSTs.[13]

From such a brief review it is clear that, although the GSTs of maize are relatively well characterised, many questions pertaining to their importance in herbicide detoxification remain unanswered. In particular our knowledge of the range of GST activities toward herbicides and other xenobiotic substrates present in maize as a whole, and in the various individual isoenzymes identified over the last ten years remains fragmentary. Also most of the studies to date have concentrated on the GSTs in the foliage and, with some exceptions,[14] other tissues have been largely ignored.

We now report on the spectrum of GST activities toward herbicides and xenobiotics known to be substrates of the various classes of GSTs in animals,[15] in crude extracts from the shoots and roots of etiolated maize treated with and without the safener dichlormid. We have also purified the GST isoenzymes containing the GST I subunit present in the roots of safener-treated maize seedlings and individually characterised their portfolio of activities.

2 MATERIALS AND METHODS

2.1 Plant material and growth conditions

Seeds of maize (*Zea mays* L.) cv. Pioneer 3394 were supplied by Pioneer Seeds UK Ltd, Hartford, Northwich, UK and cv. Artus by Sharp International, Avonmouth, UK and were washed briefly in tap water to remove any seed dressing prior to use. Seed was then either imbibed for 1 h in tap water or in an aqueous solution of dichlormid (10 μg ml^{-1}) prepared by dissolving dichlormid in acetone (5 mg ml^{-1}) and then mixing (2 + 998 by volume) with water. Seeds were then sown onto vermiculite pre-wetted and subsequently watered with either water or 5 μg ml^{-1} dichlormid, +1 ml litre^{-1} acetone in water, and then either grown in the light at 25°C with a 16-h photoperiod and light intensity of 150 μmol m^{-2} s^{-1} or grown in a light-proof cupboard at ambient laboratory temperature. At 10 days after sowing, shoots were harvested by cutting the base of the stems and roots were carefully washed free of any vermiculite using distilled water and then blotted dry. After weighing, the tissues were either extracted directly or frozen in liquid nitrogen and stored at −80°C prior to use.

2.2 Chemicals

Herbicides and their respective glutathione conjugates were obtained or prepared as described previously.[16] All other GST substrates were purchased from either

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the Sigma or Aldrich Chemical Companies. Dichlormid was obtained from Rhône-Poulenc Agriculture Ltd.

2.3 Assay of GST activities

GST activities toward herbicide substrates were determined by quantifying the GSH conjugates formed using reversed-phase HPLC.[16] GST activities toward 1-chloro-2,4-dinitrobenzene (CDNB), 1,2-dichloro-4-nitrobenzene (DCNB), 1,2-epoxy-3-(4-nitrophenoxy) propane (ENPP), *trans*-4-phenyl-3-buten-2-one (PBO), 4-nitrobenzyl chloride (NBC), 4-nitrophenethyl bromide (NPB), bromosulfophthalein and ethacrynic acid were determined using published spectrophotometric assays,[15] with the modification that all assays were carried out in a total volume of 1 ml with GSH present at a final concentration of 5 mM. Activities toward the α,β-unsaturated aldehydes and isothiocyanates were also determined spectrophotometrically using vinyl pyridine and crotonaldehyde[17] and benzylisothiocyanate[18] as the respective substrates. In all cases GST activities were expressed in katals (mole of product formed s^{-1}). GSH peroxidase activity was determined with cumene hydroperoxide as substrate using an NADPH-coupled assay.[19] Protein content in all assays was determined using the Bio Rad dye-binding assay as detailed by the manufacturer with bovine gamma-globulin being used as the reference protein.

2.4 Purification of GSTs

All purification steps were carried out at 4°C using Pharmacia GradiFrac protein purification equipment. Plant tissue was homogenised in a Waring blender in 3 volumes of Tris HCl (0·1 M; pH 7·5) containing EDTA (2 mM), 2-mercaptoethanol (14 mM) and polyvinyl-polypyrrolidone (50 g kg^{-1}). After straining through nylon mesh (pore size 10 μm) the extract was centrifuged (17 000g, 30 min) and ammonium sulphate slowly added to the supernatant to 80% saturation. The precipitated protein was then collected by centrifugation as above and resuspended in Buffer A [potassium phosphate buffer (10 mM; pH 7·4) with 2-mercaptoethanol (14 mM)] containing ammonium sulphate (0·5 M). After clarification by centrifugation, the preparation was loaded onto a column of phenyl sepharose (Pharmacia, total column volume 35 ml) at a flow rate of 2 ml min^{-1}. The eluant was monitored for UV absorbance at 280 nm and the column washed with buffer A containing ammonium sulphate (0·5 M) until all unbound protein had been washed through. The column was then washed with buffer A at 2 ml min^{-1} and 10-ml fractions collected and assayed for GST activity with CDNB as substrate. The wash with buffer A was continued until the UV absorbance had returned to basal levels, indicating that protein elution under these conditions was complete. Finally the column was washed with a mixture of buffer A and ethylene glycol (1 + 1 by volume) containing GSH (2 mM) at 0·5 ml min^{-1} and fractions collected and assayed until no further GST activity was observed. The active fractions eluted with ethylene glycol were then pooled and applied to a fast flow Q-sepharose column (total volume 6 ml) and washed with buffer B [Tris HCl (20 mM; pH 7·8) containing 2-mercaptoethanol (14 mM)] at a flow rate of 0·5 ml min^{-1} to remove ethylene glycol. The GSTs were then eluted with buffer B containing sodium chloride (0·25 M) and collected in 2-ml fractions. Pooled active fractions were dialysed for 16 h against potassium phosphate buffer (10 mM; pH 6·0) and then applied to an Orange A affinity column (total volume 6 ml, Amicon) at a flow rate of 1 ml min^{-1}. After the sample had passed through the column it was reapplied to maximise binding. The column was then washed with potassium phosphate buffer (50 mM; pH 7·0) as above until the UV absorbance of the eluant indicated that all non-specifically bound protein had been eluted. The GST-containing fraction was selectively recovered with potassium phosphate buffer (50 mM; pH 7·0) containing GSH (2 mM). Finally sodium chloride (1 mM) was added to this buffer to ensure complete recovery of any proteins which were tightly bound.

Isoenzymes of GST were resolved on Q-sepharose using either open columns (total volume 6 ml) or prepacked HiTrap Q cartridges (Pharmacia) by loading samples on in Tris HCl (20 mM; pH 7·8) and then selectively eluting the bound protein with a linear gradient of 25 column volumes of increasing sodium chloride up to a final concentration of 0·25 M. Fractions (100% of the respective column volume) were then assayed for all GST activities of interest. At all stages the purification of the GSTs was monitored by analysing the samples by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) on 0·8 mm thick gels (12·5% acrylamide and 0·33% *N'N'*-bis-methyleneacrylamide) using a BioRad Mini-Protean apparatus as recommended by the manufacturer. Resolved peptides were visualised by silver staining using the respective Bio Rad kit. For protein sequencing purified preparations in solution were applied to ProBlott PVDF membranes and subjected to automated microsequencing on an Applied Biosystems 477A protein sequencer.

3 RESULTS

3.1 GST activities in maize seedlings treated with and without dichlormid

In initial experiments the range of GST activities toward non-herbicide xenobiotic substrates was determined in desalted crude extracts from the shoot tissue of etiolated maize seedlings (cv. Artus) treated with and without the safener dichlormid (Table 1). Activities toward the substrates were in the order CDNB >

TABLE 1
GST Activities toward Xenobiotic Substrates in Crude Extracts from Etiolated Maize (cv. Artus) Seedlings Treated with and without Dichlormid

Substrate	*Enzyme activity*[a] (*pkat mg*$^{-1}$ *protein*)	
	−Dichlormid	*+Dichlormid*
CDNB	2483 (±67)	5533 (±500)
DCNB	4 (±1)	4 (±1)
Ethacrynic acid	137 (±45)	300 (±167)
NBC	87 (±5)	173 (±5)

[a] Values given are means of duplicate determinations and the variations between the mean and the replicates.

ethacrynic acid > NBC > DCNB. Activities toward all substrates except DCNB were doubled by safener treatment. No activity above the chemical rate of conjugation could be determined towards vinyl pyridine, crotonaldehyde, benzylisothiocyanate or bromosulfophthalein in any of the samples tested. Similarly, after correcting for the chemical rate, activities toward ENPP (20(± 5) nkat g^{-1} protein) PBO (0·30(± 0·06) nkat g^{-1}) and NPB (3·8(± 2·6) nkat g^{-1}) were scarcely detectable in all samples.

The effects of dichlormid on GST activities toward the selective maize herbicides atrazine, metolachlor and alachlor and the non-selective herbicide fluorodifen were determined in both the shoot and root tissues of etiolated maize seedlings. Activities in desalted crude root and shoot extracts from the two cultivars were determined by the HPLC assay described previously after correcting for non-enzymic conjugation.[16] As there is evidence in the literature[3] that GST activities vary between maize cultivars it was of interest to determine the relative GST specific activities toward the herbicides, and their respective sensitivities to safener treatment in two differing varieties. In the roots and shoots of both cultivars (Table 2) the relative distributions of GST activities toward the herbicides were similar in untreated plants, with the exception of atrazine-conjugating activity in the root. Both cultivars had similar specific activities toward all substrates except for atrazine-specific activity, which was considerably higher in Pioneer 3394, especially in root tissue. In untreated shoots, activities were in the order metolachlor = atrazine = alachlor > fluorodifen. In the roots the order was alachlor = metolachlor > atrazine ⩾ fluorodifen. Interestingly, the GSTs with activities toward chloroacetanilides were more highly expressed in the roots than in the shoots, while the opposite appeared to be the case for the activity toward atrazine, at least for the cultivar Artus. In contrast, the specific activities of the GST conjugating fluorodifen were similar in the two tissues. Treatment of the seedlings with dichlormid also revealed some interesting differences in the relative expression of the different activities. In the shoots of both cultivars, safener treatment increased the activity towards alachlor three- to four-fold, the activity towards metolachlor and fluorodifen approximately two-fold and had a negligible effect on atrazine-conjugating activity. In the roots of both cultivars dichlormid treatment increased activities toward alachlor and metolachlor, but to a considerably lesser extent than that observed in shoots. Activity toward fluorodifen in the roots of both cultivars also increased on application of dichlormid; this increase was similar to that in shoots. However, the activity toward atrazine was increased significantly by dichlormid treatment only in Pioneer 3394, suggesting that varietal differences exist with respect to GST induction. Collectively these observations regarding the differential expression of the GST activities depending on organ type and safener treatment suggest that the regulation and substrate

TABLE 2
GST Activities toward Herbicide Substrates in the Roots and Shoots of Etiolated Seedlings of the Cultivars Artus and Pioneer 3394 Treated with and without Dichlormid

Cultivar and tissue	*(−/+) dichlormid*	*Enzyme activity*[a] (*pkat mg*$^{-1}$ *protein*)			
		Atrazine	*Alachlor*	*Metolachlor*	*Fluorodifen*
Artus					
shoot	−	1·7 (±0·3)	1·3 (±0·4)	2·4 (±0·9)	0·6 (±0·2)
	+	1·9 (±0·3)	4·6 (±2·0)	5·5 (±0·9)	1·5 (±0·1)
root	−	0·8 (±0·2)	14·1 (±1·5)	9·2 (±1·9)	0·7 (±0·3)
	+	0·5 (±0·2)	16·1 (±1·7)	17·2 (±3·2)	2·0 (±0·2)
Pioneer 3394					
shoot	−	2·4 (±0·2)	1·5 (±1·4)	3·8 (±0·4)	0·3 (±0)
	+	3·6 (±0·8)	5·9 (±2·5)	6·5 (±0·6)	0·7 (±0·1)
root	−	2·3 (±0·4)	15·3 (±2·5)	12·0 (±1·2)	0·5 (±0·1)
	+	5·3 (±0·3)	20·5 (±1·0)	18·0 (±1·5)	1·2 (± 0·1)

[a] Values given are means of duplicate determinations and the variations between the mean and the replicates.

specificity of GST isoenzymes in maize is very complex, as suggested by genetic[12] and biochemical[13,20] studies. It was therefore necessary to examine the types of GST which were present in the differing organs following the various treatments and examine the specificities of purified GST isoenzymes.

3.2 Nomenclature of GSTs in maize

The current nomenclature for the GSTs involved in herbicide metabolism in maize has developed from their order of discovery combined with their relative order of elution from anion-exchange columns. While this numbering system was adequate in the original descriptions of the GSTs, its continued use is proving problematical owing to the continued discovery of new GSTs in maize, which contain subunits which have already been described. One alternative would be to classify the GSTs based on the molecular masses (Mr) of the component subunits. However, in future this could lead to some confusion in view of the similarities in molecular masses of the maize GSTs described to date and the discrepancies in the Mr calculated by SDS-PAGE and the actual Mr calculated from the coding sequence. Instead we propose a numbering system based on the order of discovery of the individual subunits, rather than the isoenzyme dimers. Thus, the 29 kDa subunit is termed GST I, the 27 kDa subunit GST II and the 26 kDa subunit GST III. This renumbering system, which is more flexible and less ambiguous than the existing classification and which will be referred to in the remainder of the paper, is summarised for the known GSTs of maize in Table 3.

3.3 Effect of dichlormid on GST isoenzymes in maize roots and shoots

Maize seedlings (cv. Artus) were grown in the dark for 10 days with and without dichlormid treatment and crude extracts from roots and shoots containing identical amounts of total protein were analysed by ion exchange chromatography on Q-sepharose to resolve major GST isoenzymes (Fig. 1). When fractions were assayed with CDNB, both roots and shoots appeared to contain two major forms of GST. The elution of the isoenzymes was very similar to that obtained with extracts from dichlormid-treated maize on DEAE sepharose[5] and Mono Q FPLC;[14] the isoenzyme eluting first appeared to be GST I/I and the second isoenzyme GST I/II. On comparing the elution profiles under conditions of identical protein loading, both untreated and dichlormid-treated roots contained higher activities toward CDNB than did the respective shoot extracts. The variation in the elution of GST I/I in unsafened and safener-treated shoot extracts resulted from the relatively low resolution of the anion-exchange column and was not otherwise significant. As described in previous studies, GST I/II made a minor contribution to the total GST activity eluting from the column in untreated shoot extracts but represented some 30% of the total activity following dichlormid treatment. Our results clearly show that safener treatment resulted in an increase in GST I/I as well as increasing GST I/II in both shoots and roots, with the relative induction being greatest in the roots. In untreated roots approximately 40% of the total GST activity toward CDNB was associated with GST I/II, demonstrating that this isoenzyme is constitutively expressed in the roots.

TABLE 3
Revised Classification System for Maize GSTs based on Subunit Identity

Old GST classification	*Subunit composition*	*New*[a] *classification*
GST I	29, 29	GST I/I
GST II	29, 27	GST I/II
GST III	26, 26	GST III/III
GST III	29, 26	GST I/III
GST IV	27, 27	GST II/II

[a] GST I = 29 kDa subunit, GST II = 27 kDa subunit, GST III = 26 kDa subunit.

3.4 Purification of GST isoenzymes in maize

Preliminary studies suggested that the roots of 10-day-old etiolated maize seedlings treated with dichlormid were the optimal source for the purification of GSTs (Table 2). Due to the availability of Pioneer 3394 this variety was used for all purification work.

To avoid the need to desalt the ammonium sulphate-precipitated crude protein extract, the hydrophobic GSTs were bound to a phenyl sepharose column. Unlike the majority of proteins, which are not retained

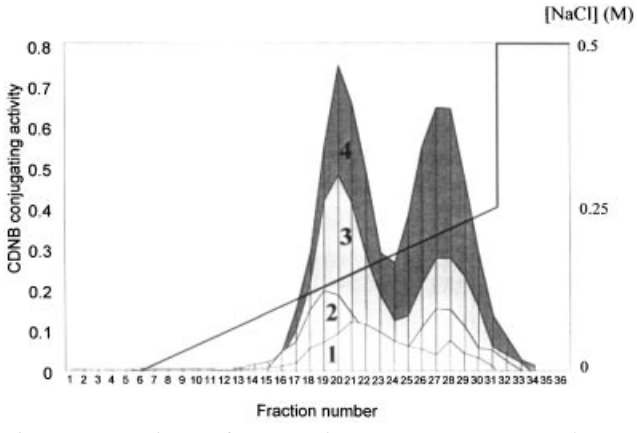

Fig. 1. Separation of GST isoenzymes on Q-sepharose present in (1) untreated shoots, (2) dichlormid-treated shoots, (3) untreated roots and (4) dichlormid-treated roots. Identical amounts of protein were loaded for all extracts and fractions assayed for GST activity toward CDNB which is expressed as change in absorbance at 340 nm over 30 s.

under the loading conditions used, GSTs eluted in two discrete fractions (Fig. 2A). The less hydrophobic fraction, representing 20–25% of the total GST activity recovered from the column, was eluted in the absence of ammonium sulphate. This fraction was termed the polar GST fraction. However the majority of the GST activity toward CDNB could only be eluted in the presence of ethylene glycol and this was termed the hydrophobic GST fraction. The further purification of the maize GSTs concentrated on the hydrophobic fraction.

After removing the ethylene glycol by anion-exchange chromatography, the hydrophobic GSTs were affinity-purified using an Orange A matrix (Fig. 2B). The Orange A ligand appears to bind to the active site of GSTs and its use as an affinity matrix for plant GSTs has been described.[3] With CDNB as substrate, 8% of the applied activity was unretained on the column (unbound fraction) while 51% was recovered in the presence of GSH. Reapplication of the unbound fraction confirmed that no further GST activity was retained on the Orange A agarose and that it contained GSTs with differing affinities to the ligand from those present in the bound fraction. An account of the characterisation of the GSTs in this non-retained fraction will be reported separately.

The majority of the GST activity toward the herbicides alachlor, metolachlor, atrazine and fluorodifen was present in the affinity-purified preparation of the hydrophobic GSTs and this fraction was characterised in greater detail after resolving the GSTs present by ion exchange chromatography on Q-sepharose (Fig. 3). The affinity-bound final preparation contained three UV-absorbing peaks. The two major UV-absorbing peaks were clearly associated with distinct activities toward

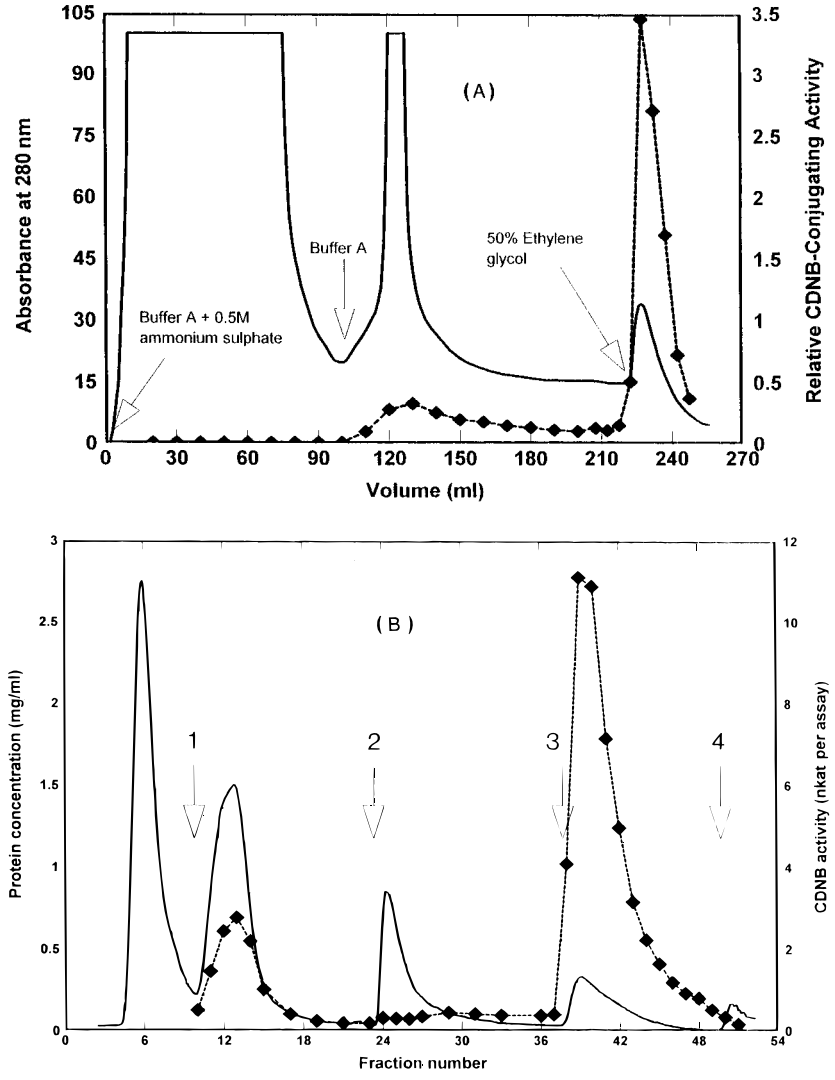

Fig. 2. Purification of GSTs from dichlormid-treated maize roots by (A) hydrophobic interaction chromatography on phenyl-sepharose eluted with the buffers indicated. (B) Affinity chromatography using an Orange A dye ligand eluted as described in the methods showing (1) the re-application of the sample, (2) a wash with 50 mM phosphate buffer, (3) a wash with 50 mM phosphate buffer containing 2 mM GSH and (4) a wash with 50 mM phosphate buffer containing 2 mM GSH and 1 M NaCl. The UV absorbance of the eluant at 280 nm is shown as an unbroken line, while fractions were assayed for GST activity toward CDNB (◆). Enzyme activity is expressed as change in absorbance at 340 nm over 30 s.

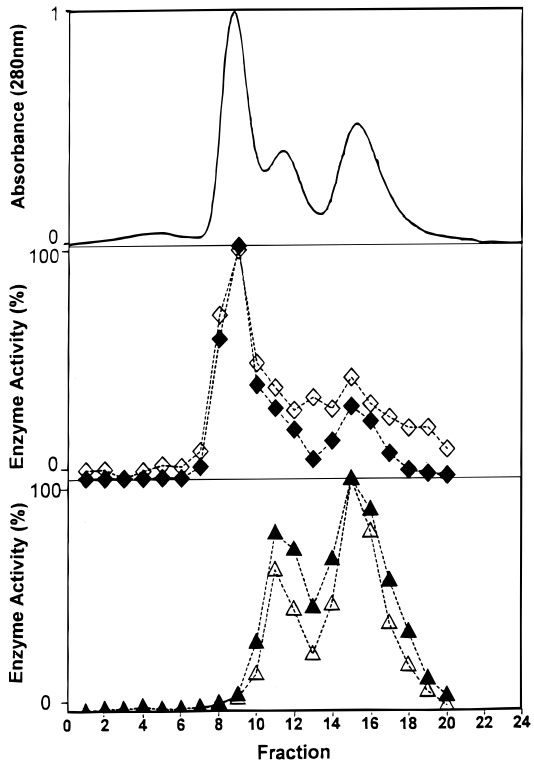

Fig. 3. Resolution on a HiTrap Q-sepharose anion-exchange cartridge of affinity-purified GST isoenzymes derived from dichlormid-treated roots with activity toward (◆) CDNB, (◇) atrazine, (△) metolachlor and (▲) fluorodifen. Enzyme activities toward each substrate are expressed as a percentage of the maximum activity determined in the eluting fractions. The UV absorbance at 280 nm of the eluant is shown as an unbroken line in the upper box.

CDNB and atrazine. However, the assaying of fractions with metolachlor and fluorodifen suggested that the minor peak eluting at the trailing edge of the first major peak contained a discrete isoenzyme with high activities toward these herbicides. To improve the resolution of this minor isoenzyme, fractions 11 and 12 were dialysed and reapplied to the HiTrap Q cartridge. In the presence of increasing salt, two UV-absorbing peaks with GST activity were clearly resolved (Fig. 4). The polypeptide composition of the individual fractions arising from anion-exchange chromatography was analysed by silver staining following SDS-PAGE (Fig. 5). The first major UV-absorbing peak showing GST activity in Fig. 3 and Fig. 4, which eluted between 100 and 125 mM sodium chloride, contained a single peptide with a molecular mass (Mr) of 29 kDa (Fig. 5). This isoenzyme appeared to be identical to the GST I/I purified in earlier reports[5,14] and this was confirmed by *N*-terminally sequencing a 5-μg sample. The amino acid sequence obtained, APMKLY, confirmed the presence of the GST I subunit.[14] Following rechromatography (Fig. 4) the second UV-absorbing peak eluting between 130 and 150 mM sodium chloride contained 29 kDa and

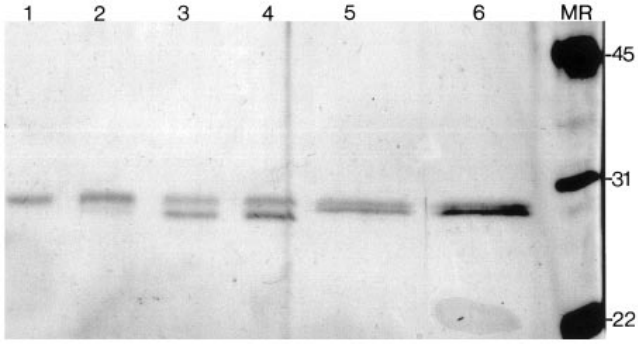

Fig. 5. SDS-PAGE analysis of GST isoenzymes resolved by ion exchange chromatography with polypeptides visualised by silver staining. Lanes 1 to 4 show fractions from the re-analysis of GST I/I and GST I/III (see Fig. 4) with lane 1 = fraction 4, lane 2 = fraction 6, lane 3 = fraction 8 and lane 4 = fraction 10. GST I/II present in fraction 15 from Fig. 3 is shown at two loadings in lane 5 (2 μg protein) and lane 6 (4 μg protein). Molecular weight markers are shown in the lane labelled MR with the molecular masses in kDa indicated.

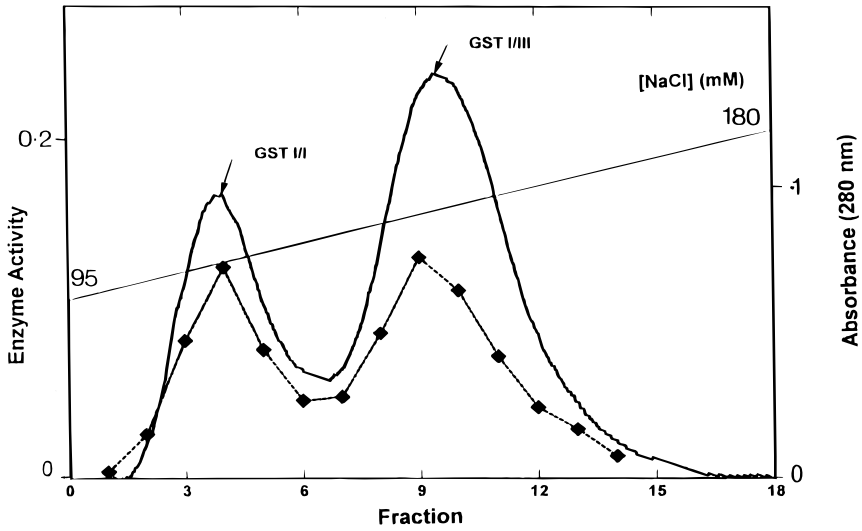

Fig. 4. Re-analysis of fractions 11 and 12 from Fig. 3 by HiTrap Q anion exchange chromatography. The protein eluting from the cartridge using the salt gradient indicated was monitored for UV absorbance as shown with the unbroken line and fractions assayed for GST activity with CDNB as substrate (◆). Enzyme activity refers to the change in absorbance at 340 nm over 30 s.

TABLE 4
Specific Activities of GSTs Purified from Dichlormid-Treated Maize Roots toward Herbicide and Xenobiotic Substrates

Substrate	*Enzyme activity (nkat mg^{-1} protein)*		
	GST I/I	*GST I/III*	*GST I/II*
Atrazine	0·11	0·07	0·07
Alachlor	1·47	1·25	3·20
Metolachlor	0·04	1·93	1·72
Fluorodifen	0·01	0·19	0·26
CDNB	1693	1240	1125
DCNB	1·10	0·18	0·24
NBC	58·17	18·67	21·00
NPB	0·57	0·28	0·31
Ethacrynic acid	27·0	19·7	25·0
Cumene hydroperoxide	ND[a]	—	3·16[b]

[a] ND = None detected.
[b] Activity expressed as change in absorbance at 366 nm min^{-1} mg^{-1} protein.

26 kDa polypeptides (Fig. 5). Sequence analysis confirmed that the 29 kDa peptide was identical to that determined in GST I, but no sequence was obtained for the 26 kDa subunit. Timmerman[3] has reported that the GSTs which bound to an Orange A affinity column contained an isoenzyme composed of a 29 kDa and 26 kDa subunit, with the smaller subunit being identified as GST III. We therefore termed this heterodimer GST I/III. The final peak of activity shown in Fig. 3, which eluted in fractions 13–18 (165–200 mM sodium chloride), contained 29 kDa and 27 kDa subunits (Fig. 5) and appeared to be GST I/II. The identity of GST I was confirmed by *N*-terminal sequencing. As reported in earlier accounts no sequence was obtained from the 27 kDa subunit which is *N*-terminally blocked in maize.[14]

3.5 Spectrum of GST activities associated with purified isoenzymes

The purified GST isoenzymes GST I/I, GST I/III and GST I/II were assayed with a range of xenobiotic and herbicide substrates and the specific activities toward each substrate are shown in Table 4. Each of the isoenzymes was shown to have distinct preferences for the xenobiotic substrates tested. GST I/I had appreciable activity toward atrazine and was active toward alachlor, but showed little activity toward metolachlor and fluorodifen. GST I/I also showed broad-ranging activities toward the other xenobiotic substrates but had no detectable activity as a glutathione peroxidase. GST I/III and GST I/II showed a similar spectrum of activities toward the various substrates, though GST I/II typically was more efficient in conjugating fluorodifen and alachlor. As compared with GST I/I, both GST I/III and GST I/II were less efficient in conjugating atrazine and the non-herbicide substrates and far more active toward fluorodifen and metolachlor.

Interestingly, the GST I/II fraction also had activity as a glutathione peroxidase with cumene hydroperoxide as substrate. As judged from SDS-PAGE analysis, the GST I/II preparation did not contain any contaminating polypeptides which could account for this peroxidase activity (Fig. 5). As the GST I subunit does not possess glutathione peroxidase activity it appears that the activity is associated with GST II and that this subunit is active as both a GST and glutathione peroxidase as has been determined for a GST in wheat flour.[19]

It was also possible to calculate the contribution each isoform made toward conjugating CDNB and each of the herbicides in the fraction of proteins eluted from the Orange A column, assuming each was purified with similar recoveries. A summary of this distribution of GST activities toward CDNB and the herbicides in the purified isoenzymes is shown in Table 5.

TABLE 5
Activities of GSTs Purified from Safener-Treated Roots as Percentage of Total Activity Eluted from Orange A column

Substrate	*Activity (% of total recovered activity)*		
	GST I/I	*GST I/III*	*GST I/II*
CDNB	53·3	18·3	28·4
Atrazine	53·1	17·5	29·4
Alachlor	32·1	12·7	55·2
Metolachlor	1·5	39·4	59·1
Fluorodifen	2·3	28·9	68·8

4 DISCUSSION

The results from the crude extracts demonstrated that the GST activities toward atrazine and fluorodifen are responsive to safener treatment in addition to the

chloroacetanilide-conjugating activities more commonly associated with safening in maize. The dichlormid-mediated increase in activity toward atrazine was only observed in one cultivar and was restricted mainly to the roots. The failure of dichlormid to elevate significantly atrazine-detoxifying GSTs in shoots may explain why this safener does not protect maize from chloro-*s*-triazine herbicides. In contrast, dichlormid has a well-characterised activity as an inducer of GSTs active in chloroacetanilide detoxification and safens these compounds.[3,14,20] Our results also suggest that dichlormid may give some protection against the non-selective herbicide fluorodifen, since following dichlormid treatment the respective GST underwent a two-fold enhancement in activity. Variations in GST specific activities toward the various herbicide substrates in root and shoot extracts suggested that the expression of the corresponding isoenzymes is partly organ-specific. Activities toward the chloroacetanilides and fluorodifen were greater in the roots than in the shoots, while the opposite was the case for atrazine. Similarly, we have recently demonstrated that GSTs with activity toward atrazine appear to be more sensitive to growth and development than do the other GST activities in young developing maize shoots.[16]

Analysis of the GST isoenzymes present in crude extracts by anion-exchange chromatography suggested a similar range of isoenzymes to that reported in similar preparations from dichlormid-treated maize.[5,13] However, we did not observe the additional GST isoenzyme GST II/II which elutes after GST I/II and is reported to be present in benoxacor-treated maize seedlings[20] and the tassels of dichlormid-treated maize plants.[14] The absence of this additional isoenzyme in the crude extracts from safener-treated maize probably results from the use of CDNB as the substrate to locate the isoenzymes, since the additional isoenzyme shows very little activity toward this substrate. From the isoenzyme profiles it would appear that the regulation of GSTs is partly organ-dependent. As described previously, GST I/I is the major constitutive GST in both roots and shoots.[5,14] Our results also suggest that GST I/II is expressed constitutively at significant levels in the root tissue but at very low levels in shoot tissue of etiolated seedlings. Similarly, in light-grown plants, the GST II subunit is constitutively expressed in the roots but not in the foliage, where its accumulation is dependent on safener application.[14]

As described in a number of reports,[5,14] treatment with dichlormid caused a significant increase in the specific activity of GST I/II. However, by applying equivalent quantities of total protein to the ion-exchange column, we also observed a major increase in the content of GST I/I in both the roots and shoots, which has not been reported previously. Transcripts encoding GST I have previously been shown to accumulate in safener-treated maize[11,21] but the increases in the GST I/I enzyme following dichlormid treatment have not been described.

When using hydrophobic interaction chromatography, the GSTs eluted as a minor polar form in the absence of salt and a major hydrophobic form, requiring ethylene glycol to achieve elution. This result suggested that two distinct groups of GSTs, differing in their hydrophobicities, were present. However, purification of the more polar fraction suggested that it largely consisted of GST I and GST II subunits indicative of the GST I/II dimer (results not shown). The reason for this partitioning of GSTs into two fractions differing in hydrophobicity remains unclear. One explanation is that a proportion of the GSTs associate with other proteins during hydrophobic interaction chromatography and alter the chromatographic properties of the enzyme. Another explanation is that the two GST populations differ in their post-translational modifications which alter relative hydrophobicities.[1]

The division of GSTs into isoenzymes which would bind to the Orange A affinity column and those which would not was also significant. Similar chromatographic behaviour on this matrix has been reported previously for maize GSTs.[3] Significantly, all the retained forms contain the GST I subunit which suggests that this is the subunit form which specifically binds to the triazine dye. Such an observation may explain why the GST II/II isoenzyme, which has been reported to be a major isoenzyme in safener-treated maize extracts,[9,14] was not observed in our affinity-purified preparation, as it would have not bound to the Orange A column. Analysis of the affinity-purified GSTs on Q-sepharose columns resolved three isoenzymes, in the order GST I/I, GST I/III and GST I/II. GST I/I and GST I/II are well described[5,9,14] but GST I/III has previously been ill-defined, with only a passing reference to its presence among the isoenzymes retained on an Orange A column.[3] The identity of the maize isoenzymes containing the GST III subunits has never been well-defined. An antibody raised to the GST I/III heterodimer indicated that the GST III subunit eluted with GST I/II on ion-exchange columns, though the subunit composition of the GST III-containing dimer was not reported.[19] In contrast, it has been reported that the major GST containing GST III subunits in maize exists as the GST III/III homodimer, although unfortunately the chromatographic behaviour of this isoenzyme relative to the other GSTs was not reported.[6] The results of our study suggest that the GST III subunit can form dimers with the GST I subunit in a manner analogous to that seen with the GST II subunit. The relative importance of this heterodimer remains to be determined, since, as discussed above, the affinity matrix appears to have selectively bound GST I-containing isoenzymes and the GST III/III isoenzyme may exist, as would be suggested by the studies of O'Connell *et al.*,[6] in the unretained fraction.

The substrate specificities of the three purified GST isoenzymes can be summarised as follows. GST I/I showed the broadest range of specificities of any of the isoenzymes tested to the herbicide and xenobiotic substrates. This isoenzyme showed significantly higher activity toward atrazine than the other isoenzymes, suggesting that atrazine conjugation was a feature of the GST I subunit. In view of the uncertain nature of the GSTs catalysing atrazine detoxification in maize, this observation was of particular interest. The current literature is ambiguous on this point, with one report suggesting that GST I/I did show some activity toward atrazine,[3] while a more recent account indicated that this isoenzyme had no activity toward this substrate.[14] When a GST I coding sequence was assembled from synthetic oligonucleotides and expressed in recombinant bacteria, the resulting enzyme was shown to have activity toward atrazine.[22] Our results would suggest that GST I/I can account for only a minor proportion of the total atrazine-detoxifying GST capacity of maize and it is likely that the specific atrazine-detoxifying GST remains to be discovered in the non-retained fraction from the Orange A column.[3] GST I/I was also of interest, as, under saturating substrate concentrations, although less active than the other GST isoenzymes, it catalysed the conjugation of alachlor some 40 times more efficiently than the conjugation of the related chloroacetanilide, metolachlor.

GST I/III and GST I/II showed a similar spectrum of activities toward the various substrates, though GST I/II typically was considerably more efficient in conjugating fluorodifen and alachlor. These results confirm previous suggestions that the GST II and GST III subunits show a greater catalytic efficiency in conjugating chloroacetanilides and diphenyl ether herbicides than the GST I subunit[6,14] and show higher activity toward alachlor than to metolachlor.[6]

It was also of interest that the GST II subunit showed activity as a glutathione peroxidase. Glutathione peroxidases with GST activity are important in reducing lipid hydroperoxides and play an important role in counteracting oxidative stress.[19] GST II in maize is induced in response to safener treatment, phytotoxic injury by salicylic acid and during senescence.[11] It will now be of interest to determine whether or not the induction of GST II in all these cases results from its protective role as a glutathione peroxidase and is a response to the accumulation of naturally occurring hydroperoxides formed during oxidative stress.

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